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Protection Against Herpes B Virus Infection in Rabbits With a Recombinant Vaccinia Virus Expressing Glycoprotein D

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Herpes B virus infects naturally monkeys of the macaque genus in whom it can cause recurrent oral and genital lesions. However, when the virus infects humans it causes a neurological illness with a high case fatality rate. Successful treatment is possible but this depends on diagnosis prior to the onset of respiratory arrest, and fatalities over the last 10 years have been the result of late or no diagnostic data on which to base anti-viral intervention. An effective vaccine would be an ideal way to combat the risk of herpes B virus disease in humans working with potentially infected monkeys or their tissues. A recombinant vaccinia virus expressing herpes B virus glycoprotein D (gD) was constructed and rabbits inoculated with the chimeric virus were tested for immunoglobulin responses to herpes B virus by virus neutralisation, ELISA and Western blot analyses. Anti-gD humoral responses were detected in all vaccinated animals by ELISA and Western blot but neutralising antibody was not detected prior to challenge with herpes B virus. Non-vaccinated rabbits died within 8 days of challenge while 10/11 vaccinated animals were protected against herpes B virus disease. No antibodies to herpes B virus proteins other than gD were detectable in surviving animals, suggesting minimal herpes B virus replication post challenge. Autopsies were carried out on 4/10 rabbits which had remained healthy at 31 days post challenge and the dorsal root ganglia adjacent to the inoculation site were removed. Attempts to detect herpes B virus DNA by PCR followed by hybridisation proved negative suggesting protection against latent herpes B virus infection. ***J. Med. Virol. 57:47–56, 1999.***

KEY WORDS: Cercopithecine herpesvirus 1; recombinant vaccine; protective immune response

INTRODUCTION

More than thirty-five different herpesviruses of primates have been isolated and identified [McCarthy and Tosolini, 1975; Eberle and Hilliard, 1995]. Of these, herpes B virus poses the greatest threat to the health of monkey handlers [Weigler, 1992]. Formally known as cercopithecine herpesvirus 1 [Roizman et al., 1992], herpes B virus naturally infects monkeys of the macaque genus causing recurrent oral or genital lesions which clear spontaneously. However, accidental transmission to man from monkey bites or via infected tissues or fluids results in acute ascending encephalomyelitis which was fatal in almost 70% of cases reported prior to 1995, due to late diagnosis resulting in treatment being unsuccessful [Holmes et al., 1995]. Despite the serological cross-reaction between HSV and herpes B virus, humans who were apparently HSV seropositive were not protected against fatal herpes B virus infection [Weigler, 1992].

Several approaches have been developed to control human infection with herpes B virus. Preventative measures include the use of herpes B virus free macaques for biomedical research, and guidelines for handling macaques [Simian Virus Committee, 1985; DiGiacomo, 1988; B virus Working Group, 1988; Ward & Hilliard, 1994]. Early diagnosis is of paramount importance in treating human cases [Holmes et al., 1990] and although anti-viral therapy reduces mortality by nearly 100% if it is initiated prior to the onset of respiratory arrest, long-term suppressive treatment with acyclovir is required in order to avoid reactivation of latent virus [Holmes et al., 1995]. Antibody-based de-

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tection is complicated by the close serological relationship between herpes B virus and herpes simplex virus (HSV) which is reflected at the molecular level [Eberle and Hilliard, 1995]. The development of monoclonal antibodies [Cropper et al., 1992] and PCR-based methods for detecting herpes B virus in clinical specimens should facilitate earlier and more specific diagnosis [Scinicariello et al., 1993; Scinicariello et al., 1993a; Slomka and Brown, 1995].

An effective herpes B virus vaccine for human use could eliminate the risk of herpes B virus infection in vaccinated individuals and would be a significant addition to existing medical countermeasures. A vaccine for veterinary use could also have an important role in developing herpes B virus free macaque colonies, since many of the non-human primates used for biomedical research are still wild caught animals. Only one attempt to develop a vaccine has hitherto been reported. The vaccine consisted of formalin-inactivated virus, prepared in rabbit kidney cell cultures, and was used experimentally to immunize 300 volunteers [Hull and Nash, 1960; Hull et al., 1962]. However, the vaccine was a poor immunogen: 20% of the individuals who received it did not respond even after repeated booster doses at 3–6 month intervals [Hull, 1971], and it was never licensed for general use.

Extensive studies on this pathogen are impractical due to the necessity of carrying out in vitro manipulations under ACDP Category III containment and in vivo research under ACDP Category IV containment [Advisory Committee on Dangerous Pathogens, 1995]. Insufficient knowledge concerning the properties of herpes B virus, particularly with respect to latent infections, precludes the development of an attenuated strain of the virus for vaccination purposes. We previously reported cloning of the gene encoding herpes B virus glycoprotein D with a view to constructing a recombinant vaccine against this virus [Bennett et al., 1992]. Recent progress in HSV vaccine development has included the use of recombinant vaccinia virus (reviewed by McKenzie and Straus, 1996). Cremer et al. [1985] showed that a vaccinia/HSV-1 gD recombinant protected against lethal challenge by HSV-1 or HSV-2 and the establishment of latent infections by HSV-1. Blacklaws et al. [1990] concurred with this data and showed that a vaccinia/HSV-1 gD recombinant performed better than recombinants expressing gB, gC, gE, gG, gI, and gH in terms of antibody response and protective immunity in a mouse model. More recently, primary and latent infections with HSV-2 have been shown to be limited by DNA vaccines expressing HSV-1 gD [Bourne et al., 1996] and HSV-2 gD [Bourne et al., 1996a; McClements et al., 1996]. Chabalgoity et al. [1996] also demonstrated protection against HSV using Salmonella which expressed a fusion protein of tetanus toxin fragment C and an immunogenic peptide from HSV gD. In order to develop an effective herpes B virus vaccine, a recombinant vaccinia virus was constructed which expressed the gD gene of herpes B virus. The ability of the recombinant virus to elicit protective immune responses against herpes B virus disease was investigated.

MATERIALS AND METHODS

Cells and Virus

The WR strain of vaccinia virus was used for making recombinant virus. Human TK$^-$ 143B cells, used for transfection and selection of recombinant virus, were obtained from the European Collection of Animal Cell Cultures and grown in Eagle's medium containing 10% foetal calf serum (FCS; Life Technologies, Bedfordshire, UK) and 25 μg/ml of 5-bromodeoxyuridine (Sigma). Stocks of vaccinia viruses were produced and titrated using CV-1 cells grown in Eagle's medium containing 10% FCS.

The strain of herpes B virus used in this work has been designated prototypic herpes B virus [Wall et al., 1989] and was termed Cyno 2. The prototypic strain was an oral isolate from a cynomolgus monkey [Vizozo, 1975]. Herpes B virus was inoculated into simian Vero E6 cells grown in medium 199 supplemented with 10% FCS, and virus was recovered as described for HSV antigen, but without sonication [Slomka et al., 1995a].

Growth of herpes B virus in tissue culture was performed under ACDP Category III containment conditions.

Construction of Recombinant Vaccinia Virus Expressing Herpes B Virus gD

The herpes B virus gD gene was subcloned from the plasmid pSK$^+$2.6 [Bennett et al., 1992] into a shuttle vector plasmid, p1107. p1107 was derived from the plasmid pGS20 [Mackett et al., 1984] by inserting the 19K early vaccinia promoter upstream of the *Ecogpt* gene [Carroll, 1993]. Expression of the *Ecogpt* gene allows dominant selection of recombinant viruses [Isaacs et al., 1990]. The 1.8 kbp *Xho* I fragment containing the gD gene was isolated from pSK$^+$2.6, recessed 3′ ends were filled using the Klenow enzyme, and the fragment was cloned into the *Sma* I site of p1107. The resulting plasmid, pAK2, was used to transfect vaccinia virus-infected HuTK$^-$ 143B cells using Lipofectin reagent according to the manufacturer's instructions (Life Technologies, Bedfordshire, UK). Recombinant viruses were selected by passaging twice in medium containing mycophenolic acid (25 μg/ml), xanthine (250 μg/ml) and hypoxanthine (15 μg/ml). Recombinant viruses were designated vBVgD and were subjected to three rounds of plaque-purification before preparation of stocks for further use.

Protection Studies Using vBVgD

New Zealand White female rabbits, Albino strain, 1.5–2.0 kg were obtained from Froxfield Farms UK

Ltd. Rabbits were divided randomly into four groups. Group 1 (3 rabbits) received a mock vaccination of PBS. Group 2 (2 rabbits) received 10^8 PFU of vaccinia strain WR. Groups 3 and 4 (6 rabbits each) received 10^8 PFU of recombinant virus vBVgD. Group 4 received a booster dose of 10^8 PFU vBVgD after 4 weeks. The rabbits were immunised by dermal scarification of the upper dorsal region. Whole blood samples were taken from each animal at weekly intervals following immunisation until 30 days post challenge, and the serum was stored at −70°C. One rabbit in group 3 sustained a vertebral fracture 7 days after immunisation and euthanasia was undertaken. All animals were challenged 66 days after the primary immunisation by subcutaneous injection of 100 PFU of herpes B virus strain Cyno 2 [Vizozo, 1975]. The rabbits were subsequently monitored for signs of disease over a four week period. Oral, vaginal, eye, and inoculation site swabs were taken at death from animals in groups 1 and 2. The dorsal route ganglia adjacent to the inoculation site were taken from 2 animals in group 3 and 2 animals in group 4 at 31 days post challenge.

The animals were maintained under ACDP Category IV containment conditions throughout the study and Home Office guidelines on the use of experimental animals were strictly adhered to.

ELISA

Herpes B virus antigen was prepared by infecting twenty 80 cm^2 flasks of BHK-21 cells at a multiplicity of infection of 0.1 and incubating at 37°C for 48 hours. Monolayers were washed and scraped into PBS. Cells were pelleted at 2000 rpm for 5 mins, and the pellet was frozen and thawed three times. Herpes B virus infectivity was inactivated by gamma irradiation (3.5 Mega Rads). The cell pellet (21 ml) was solubilised by addition of 9 ml 33% glycerol, 1.7% sodium deoxycholate, 1.7% NP40, and aliquots were stored at −70°C.

Herpes B virus antigen was diluted 1:100 in 10 mM Tris-HCl (pH 7.5) and used to coat wells of a microtitre plate (Grenier). After overnight incubation at 4°C, the wells were washed with PBS and incubated with 200 μl 10% FCS in PBS for 1 hr at 37 °C to inhibit non-specific binding. The wells were washed and 100 μl of rabbit sera was added in diluent (10% FCS, 0.1% Tween 20 in PBS). Control serum was polyclonal anti-B virus, prepared using gamma irradiated herpes B virus strain Cyno 2 [Cropper et al., 1992] which was inoculated into rabbits as described [Slomka et al., 1995b]. After 1 hr incubation at 37°C, the wells were washed four times with 0.2% Tween 20 in PBS. Anti-rabbit horseradish peroxidase conjugated immunoglobulin (Dako) was added at a dilution of 1:1000 and incubated for 1 hr at 37°C. Wells were washed four times as before and finally twice with PBS. Tetramethylbenzidine solution was used for colour development and readings were taken at 450 nm as described [Harlow and Lane, 1988]. Sera were diluted two-fold from 1:20 to 1:40960. The highest dilution at which a given sample remained positive ($OD_{450} > 0.270$, the mean of negative sera plus 3 standard deviations) was noted as the serum titre.

Western Blot Assay of Herpes B Virus Humoral Responses

E6 cell lysates (infected with herpes B virus or uninfected control) were prepared for polyacrylamide gel electrophoresis (PAGE) and electroblotted to PVDF membranes (Amersham) as described [Slomka et al., 1995b]. Rabbit sera was diluted to 1:200 in PBS containing 10% non-fat milk (Marvel) and 0.1% Tween 20. Membranes were incubated with rabbit sera, washed, and incubated with anti-rabbit horseradish peroxidase conjugate (Dako) diluted 1:1000. Immune detection was performed using enhanced chemiluminescence (Amersham) according to the manufacturer's protocol.

Virus Neutralisation

The level of neutralising herpes B virus antibody was determined as described previously [Lees et al., 1991]. Briefly, rabbit serum was heat inactivated at 56°C for 30 minutes and incubated with guinea pig serum (as a source of complement) and 100 $TCID_{50}$ of herpes B virus (range 30–300 $TCID_{50}$) for 4 hours at 37°C. Residual non-neutralised virus was titrated by plaque assay in Vero cells and the neutralisation titre was taken as the reciprocal of the serum dilution giving 50% plaque reduction.

Virus Isolation

Confluent Vero E6 cells in 25 cm^2 flasks were inoculated with swab samples obtained from the eye, mouth, vagina, and inoculation site of rabbits which had been inoculated with herpes B virus. The cells were incubated for 2 weeks in maintenance medium and examined daily for cytopathic effect.

"Hot-start" PCR of Herpes B Virus DNA

Primers which amplify a 188 bp region of the herpes B virus Cyno 2 genome were used to test the rabbit swab specimens as described previously [Slomka et al., 1993]. Amplification conditions were modified such that reactions contained 40 pmoles of each primer, 60 mM Tris-HCl (pH 9.0), 15 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 200 μM dNTPs. Each 50 μl reaction was partitioned by addition of a wax bead (GEM 100, Perkin Elmer; Chou et al., 1992) according to manufacturer's instructions. The swab samples were heat-inactivated and 10 μl was added per reaction. Amplification consisted of an initial 1 min denaturation step at 95°C followed by 35 cycles of 1 min at 95 °C, 1 min at 57°C, 1 min at 72°C. The final elongation step at 72°C was extended to 5 mins. The detection limit for this procedure was found to be 4×10^{-17} g of control DNA sample [herpes B virus *Bam* HI fragment "g", Harrington et al., 1992], which corresponds to approximately 8 molecules of herpes B virus DNA. No improvement in the sensitivity of this assay was obtained when DNA was extracted from swab samples prior to PCR.

"Hot-start" Nested PCR for Detection of Herpes B Virus in Rabbit Ganglia

Primers which span a 512 bp region of herpes B virus DNA were used to initially amplify total DNA specimens extracted from rabbit ganglia [Slomka et al., 1993]. A wax bead was added as before to partition the reaction. Extracted ganglion DNA (250 ng) was added as a 10 μl volume. The 50 μl reaction contained 40 pmoles of each primer, 60 mM Tris-HCl (pH 10.0), 15 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 200 μM dNTPs. Amplification consisted of an initial 1 min denaturation step at 95°C followed by 30 cycles of 1 min at 95°C, 1 min at 52°C, 1 min 45 seconds at 72°C with a final elongation step at 72°C for 5 mins. An aliquot of 1.25 μl of product was added to a "hot start" PCR reaction mix and amplified for 30 cycles to produce the 188 bp amplicon. Because of the hazards associated with removal of ganglia from unvaccinated rabbits infected with herpes B virus, it was decided not to obtain such a sample for use as a positive control. To determine the detection limit of this assay, reconstruction experiments were performed in which dilutions of a 9.6 kbp *Bam* HI fragment of herpes B virus DNA [fragment "g", Harrington et al., 1992] were mixed with 250 ng of total E6 cell (uninfected) DNA and amplified by the modified PCR. A detection limit of 4×10^{-17} ng of herpes B virus *Bam* HI "g" (approximately 8 molecules of herpes B virus DNA) was obtained by ethidium bromide staining of PCR product.

Detection of Amplified Herpes B Virus DNA by Oligomer Hybridisation

Amplification products were electrophoresed through 3.5% agarose and transferred to Hybond-N plus membranes (Amersham) by Southern blotting. An oligonucleotide probe of 31 bp [Slomka et al., 1993] was 3′ end-labelled with digoxygenin by terminal transferase using a commercially available kit (Boehringer). Hybridisation was carried out at 52°C for 16 hours using a probe concentration of 3 pmoles/ml. Membranes were washed twice for 5 minutes at room temperature in 2XSSC, 0.1% SDS, followed by two washes for 15 minutes in 0.1XSSC, 0.1% SDS at 50°C. Chemiluminescent detection of hybrids was performed using alkaline phosphatase conjugated anti-digoxygenin, diluted to 1:10,000 and CSPD substrate (Boehringer).

RESULTS

Expression of Herpes B Virus gD by Vaccinia Virus

We had cloned previously the gene encoding herpes B virus gD and had shown it to be homologous to glycoprotein D genes identified in other herpesviruses at the level of nucleotide and predicted protein sequences [Bennett et al., 1992]. A recombinant vaccinia virus expressing herpes B virus gD was constructed in which the gD gene was inserted downstream of a constitutive vaccinia 7.5K promoter at the thymidine kinase locus of vaccinia strain WR. Expression of herpes B virus gD

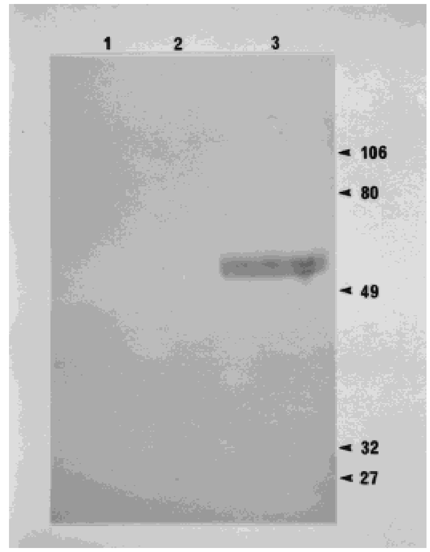

Fig. 1. Expression of herpes B virus glycoprotein D by recombinant vaccinia virus. Virus-infected CV-1 cells were boiled in Laemmli buffer, resolved on an 8% SDS-polyacrylamide gel and transferred to a PVDF membrane which was probed with rabbit antiserum to B virus. The positions and sizes (kDa) of protein markers run in parallel, are indicated. Lane 1, uninfected CV-1 cells; lane 2, CV-1 cells infected with vaccinia strain WR; lane 3, CV-1 cells infected with vBVgD.

was detected by Western blotting of vBVgD-infected cells using polyclonal rabbit-derived antiserum to herpes B virus (Fig. 1). The antiserum detected a protein of approximately 57 kDa although the mass of the unmodified protein as predicted from the amino acid sequence data is expected to be 42,646 Da. Eberle et al. [1995] have shown that herpes B virus gD is more closely related to the African simian virus SA8 and the baboon alphaherpesvirus *Herpesvirus papio 2* than to the herpes simplex virus gD proteins. Correspondingly, antisera to HSV-1 and SA8 gD glycoproteins recognise a protein of about 60 kDa in HSV-1, HSV-2, herpes B virus and SA8 infected cells [Eberle et al., 1989], whereas the predicted mass of these proteins is about 43 kDa. This discrepancy in molecular mass has also been observed with gD homologues in HSV-1 [Wachsman et al., 1987], pseudorabies virus [Petrovskis et al., 1986] and bovine herpesvirus type 1 [Tikoo et al., 1990], and may be accounted for by the addition of N- and O-linked carbohydrate during post-translational processing, or by secondary structure of the protein.

Protection of Rabbits Against Herpes B Virus Disease

All control animals (groups 1 and 2) died of B virus disease on day 8 post-challenge (Table I). Of 11 rabbits immunized with vBVgD and challenged with herpes B virus, 10 rabbits survived with no signs of ill health over a 30 day observation period. One immunised rabbit from group 4 remained healthy until day 17 when signs of reduced food intake were observed. On day 22 the animal showed a slight rear limb paralysis and died 24 hours later.

TABLE I. Summary of Results Obtained From Herpes B Virus Protection Studies

Group	Number of rabbits	Vaccination	Primary vaccination	Secondary vaccination	Challenge	Number of survivors
1	3	PBS	Day 0	None	Day 66	0/3[a]
2	2	10^8 PFU WR	Day 0	None	Day 66	0/2[a]
3	6	10^8 PFU vBVgD	Day 0	None	Day 66	5/6[b]
4	6	10^8 PFU vBVgD	Day 0	Day 28	Day 66	5/6[c]

[a]All control animals died on day 8 post challenge.
[b]One rabbit in this group sustained a vertebral fracture 7 days after immunisation. Euthanasia was undertaken.
[c]One rabbit showed reduced intake of water and food on day 17 post-challenge, although classical signs of herpes B virus infection (paralysis at site of inoculation) were not observed. On day 22 slight rear limb paralysis was noted. The animal died on day 23 post challenge.

Serum Immunoglobulin Response

All animals were bled 2 days before challenge. Unvaccinated rabbits, all of which died at 8 days post challenge, were bled at autopsy. Surviving rabbits were bled at 31 days post challenge. The presence of antibodies to herpes B virus was determined by ELISA, Western blotting and herpes B virus neutralisation.

None of the rabbits in groups 1 (unvaccinated) and 2 (inoculated with vaccinia WR) possessed herpes B virus antibody by any of the 3 assays prior to challenge. Sera obtained post-challenge at autopsy revealed that 4 of these animals still had no detectable antibody to herpes B virus, indicating that death had occurred prior to production of a humoral response (Fig. 2). One rabbit in group 2 (no. 755) showed non-specific reactivity in WB and had a neutralisation titre of 1:4 but no response was detected by ELISA (result not shown).

All 5 rabbits in group 3 (given one dose of vBVgD) developed an anti-gD humoral response prior to challenge which was still apparent at 31 days post challenge, as shown by ELISA (Fig. 2) and Western blotting (Fig. 3). Antibodies to other herpes B virus proteins were not observed by Western blotting, suggesting that herpes B virus replication had not occurred following challenge. An antibody response to Vero cell proteins was detected in serum from some animals at pre- and post-challenge time points. However, this observation was dependent both on the exposure time of the blots and the animal from which the sera was derived and may represent non-specific cross-reaction. No herpes B virus neutralisation response was detected pre-challenge. One rabbit in group 3 (no. 763) developed a weak neutralising response (1:2) at 31 days post-challenge (Table II).

Although group 4 had received two doses of vBVgD, none had developed neutralising antibody to herpes B virus prior to challenge. Antibody to gD was detected by Western blotting before and after challenge in the 5 surviving rabbits in this group (Fig. 3). Antibodies to other herpes B virus proteins were not detectable in the 5 healthy animals at 1 month post challenge, indicating absence of B virus replication. ELISA confirmed that a herpes B virus humoral response had developed prior to challenge (Fig. 2) and in view of the Western blot findings it was apparent that this response was directed at gD. The surviving rabbits in group 4 differed serologically from group 3 in their herpes B virus neutralisation response. In group 3, all 5 rabbits had neutralisation titres of 1:2 or <2 at 31 days post-challenge, whereas in group 4, 5 out of 6 rabbits had titres ranging from 1:2–1:8 (Table II). However, the humoral response was distinct in one rabbit (no. 768) from group 4 who developed paralysis and died 23 days after challenge. ELISA showed a strong antibody response to herpes B virus on the day of the first vaccination, at a time when only one other member of group 4 had a weak response (Fig. 2). An antibody response to gD was detected by Western blotting at 10 days post challenge, but 6 days later a humoral response to other herpes B virus proteins was observed (data not shown), providing evidence of herpes B virus replication. Antibody to herpes B virus remained detectable postchallenge by ELISA, while a strong herpes B virus neutralisation titre (>1:64) was apparent at the time of autopsy (Table II).

Serological responses from the 10 protected rabbits in groups 3 and 4 were compared directly by ELISA. Sera drawn 2 days prior to challenge and 1 month post challenge were tested in this manner (Table II). There was no significant difference in herpes B virus-specific immunoglobulin levels between groups of animals given single or double vaccinations, or between titres found in pre- and post-challenge sera ($P > 0.10$ for all comparisons).

Detection of Herpes B Virus Replication

Swab samples taken at death from the non-vaccinated rabbits (groups 1 and 2) were incubated with Vero E6 cells and examined for cytopathic effect. Only one animal (no. 754) yielded infectious virus from an inoculation site swab (Table III). All other swab samples were negative for virus isolation in this assay. Swabs taken from inoculation sites were tested by modified herpes B virus PCR (188 bp amplicon). A positive result was seen in samples from 3/5 rabbits by ethidium bromide staining of the agarose gel. All 5 samples were positive following oligomer hybridisation which increased the sensitivity to under 8 molecules of DNA (Table III). No inoculation site swabs were available from any of the vaccinated rabbits.

From the 10 rabbits which had remained healthy at 31 days post herpes B virus challenge, 4 were autopsied and the dorsal root ganglia adjacent to the inoculation site removed. Duplicate nested amplification of 250 ng ganglion DNA failed to reveal the herpes B virus-specific amplicon (188 bp) either by ethidium bromide

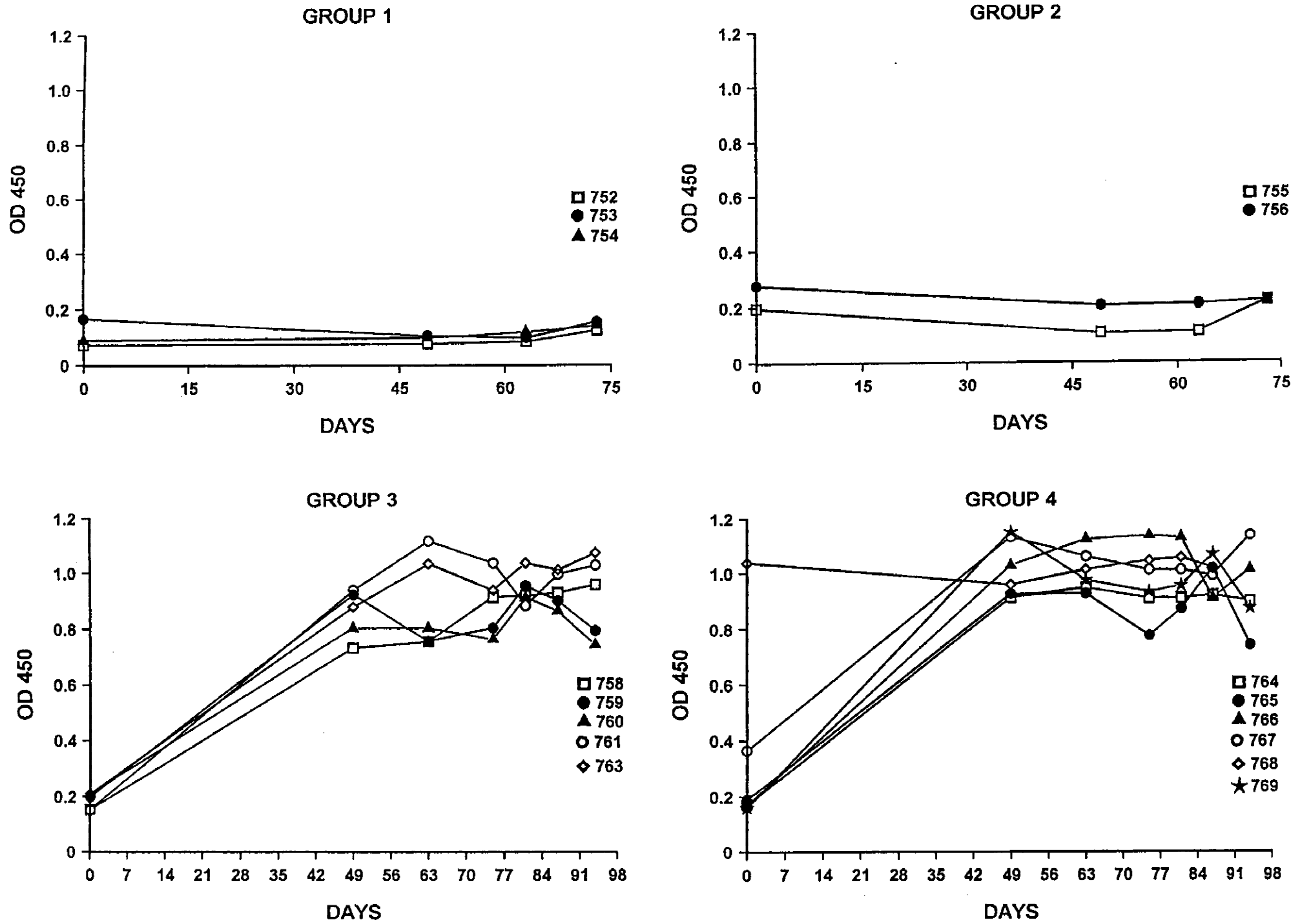

Fig. 2. Immunoglobulin response to herpes B virus determined by ELISA. Rabbit sera was diluted 1:20. The graphs show OD_{450} as a measure of herpes B virus antibody for individual rabbits in each group. Group 1 received 0.2 ml PBS on day 0. Group 2 received 10^8 PFU WR on day 0. Group 3 received 10^8 PFU vBVgD on day 0. Group 4 received 10^8 PFU vBVgD on day 0 and day 28. All animals were challenged on day 66 with B virus.

staining or by oligomer hybridisation following Southern blotting. In these 4 protected rabbits, absence of herpes B virus DNA in the ganglia suggested protection against latent herpes B virus infection, although more extensive biopsy of similar specimens is needed to confirm this observation.

DISCUSSION

A recombinant vaccinia virus has been constructed which expresses herpes B virus gD and has been shown to protect rabbits against lethal infection with herpes B virus.

Immunoglobulin titres to gD were not predictive of protection in this study. The vaccinated rabbit which succumbed to herpes B virus infection (No. 768) had an anti-gD ELISA serum titre of 1:320, whereas survivors had titres ranging from 1:160–1:640 before challenge (Table II). No boost in antibody titres to herpes B virus was seen following challenge (Fig. 2) but this may have been due to the small challenge dose used (100 PFU). The ELISA data presented in Fig. 2 concurs with Western blots (Fig. 3) where intensity of bands on the blots are similar at pre- and post-challenge time points. In the one vaccinated animal which succumbed to herpes B virus infection (No. 768), a significant boost in ELISA titre was seen (Table II), implying that the challenge virus had replicated sufficiently to raise antibody levels. There was time for a humoral response to develop in this animal due to the delayed time to death, in contrast to the unvaccinated rabbits in which death occurred prior to the production of detectable antibody levels.

It was a surprising observation that neutralising antibody was not detected prior to challenge in serum from protected animals because protection had been correlated with a neutralisation titre of 1:4 in studies using an inactivated herpes B virus vaccine [Hull et al., 1962]. Similarly, neutralising antibody had been detected following vaccination with a vaccinia recombinant expressing HSV–1 gD [Blacklaws & Nash, 1990]. The importance of using complement to achieve maximum sensitivity in detecting antibodies to herpes B virus had been established [Boulter et al., 1982] and our test not only included guinea pig serum as a source

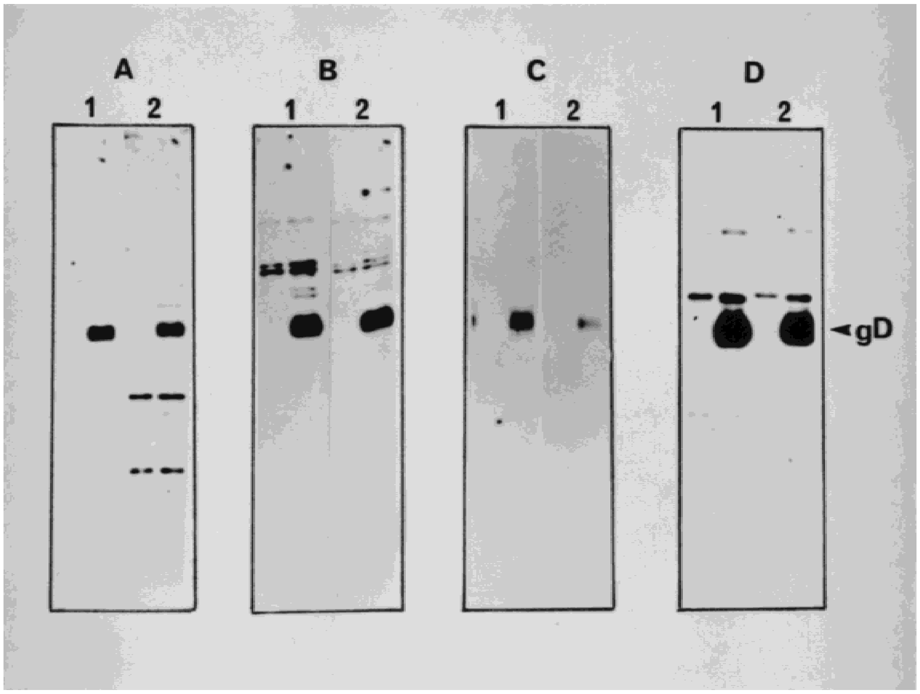

Fig. 3. Immunoglobulin responses to herpes B virus proteins in a representative sample of protected rabbits. Uninfected Vero E6 cells (left track) or Vero E6 cells infected with herpes B virus (right track) were boiled in Laemmli buffer, resolved on SDS-polyacrylamide gels and transferred to PVDF membranes. Filters were probed with antiserum from vaccinated rabbits. Panel 1 was probed with sera drawn 2 days prior to herpes B virus challenge. Panel 2 was probed with sera drawn 31 days post-challenge. A, rabbit 760 (group 3); B, rabbit 761, (group 3); C, rabbit 764 (group 4); D, rabbit 767 (group 4). The predicted migration position of herpes B virus glycoprotein D is indicated.

TABLE II. Antibody Titres in Serum From Rabbits Immunized with Vaccinia Virus Expressing Herpes B Virus Glycoprotein D

		ELISA antibody titre				Neutralisation titre at 31 days post-challenge[c]
Group	Rabbit	Pre-challenge titre[a]	Group mean[b]	31 days post-challenge[a]	Group mean[b]	
	758	80		320		<2
	759	320		320		<2
3	760	160	272 ± 196	160	352 ± 154	<2
	761	640		640		<2
	763	160		320		2
	764	160		160		<2
	765	160		160		4
4	766	640	288 ± 183	640	384 ± 213	8
	767	160		640		2
	768	320		81,920[d]		>1:64[d]
	769	320		320		8

[a]Results have been expressed as the reciprocal of the serum dilution giving $OD_{450} > 0.27$ (mean of negative sera plus 3 standard deviations).
[b]Mean values are presented with 95% confidence intervals.
[c]Results have been expressed as the reciprocal of the serum dilution giving 50% plaque reduction.
[d]Titre at autopsy 24 days post challenge, directed against multiple herpes B virus proteins, and not used for calculation of group mean.

of complement but was a more sensitive variation of the standard test [Lees et al., 1991] and had been used previously routinely and reliably to detect herpes B virus neutralising antibody. However, Cremer et al. [1985] used recombinant vaccinia virus expressing HSV-1 gD to vaccinate mice and found that the neutralising antibody response did not correlate with protection against latent HSV infection. The findings of Wachsman et al. [1987] also suggest that protection from primary cutaneous HSV disease with recombinant vaccinia virus expressing HSV-1 gD does not involve gD-induced neutralising antibody in guinea pigs. Similarly our results indicate that, despite the high virulence of herpes B virus in rabbits, neutralising antibody is not necessary for successful protective vaccination. A similar observation has been reported with a vaccinia recombinant expressing Epstein-Barr virus gp340. In this case there was no detectable anti-gp340 antibody but cotton top tamarins were protected from a dose of EBV that would induce lymphomas in unvaccinated animals [Morgan et al., 1988].

A subunit vaccine against HSV-2 has recently failed

TABLE III. PCR of Inoculation Site Swabs From Unvaccinated Rabbits Taken at Death

			Herpes B virus PCR (188 bp amplicon)	
Rabbit	Group	Herpes B virus isolation	Ethidium bromide staining	Oligomer hybridisation
752	1	−	−	+
753	1	−	+	+
754	1	+	+	+
755	2	−	+	+
756	2	−	−	+

in advanced clinical trials. Chiron/Biocine's vaccine combined HSV-2 gB and gD subunits with M59 adjuvant and, despite early clinical studies which showed the vaccine to be highly immunogenic, the formulation failed to protect against new infections and did not reduce disease severity in two large Phase III trials [Corey, 1997]. Our approach to vaccination against herpes B virus was to develop a recombinant vaccine and we have shown that 10 out of 11 animals were protected in challenge experiments following vaccination with vBVgD. Recombinant vaccines against herpesvirus infections may perform better than subunit vaccines because of their increased ability to stimulate cell-mediated as well as humoral immune responses. However, there is scope to improve the immunogenicity and level of protection afforded by the vBVgD recombinant. Co-expression of a second herpes B virus glycoprotein such as gB may increase the performance of the vaccine. In humans infected with HSV, the highest antibody titres are directed against gB [Eberle and Mou, 1983]. Expression of HSV-1 gB using vaccinia virus has been shown to elicit protective immune responses in mice against lethal HSV-1 infection [Cantin et al., 1987; Blacklaws et al., 1990]. Expression of gB in addition to gD may improve the ability of a vaccinia virus recombinant to stimulate protective immunity to herpes B virus.

Our initial studies show that herpes B virus was not found in the dorsal root ganglia adjacent to the inoculation site in the vaccinated rabbits which were screened. Herpes B virus causes a recurrent infection in monkeys, and there are anecdotal reports which suggest that latency and reactivation can also occur in humans. In a serological study of humans who had frequent contact with macaques, no cases of herpes B virus seroconversion were identified [Freifeld et al., 1995], but the possibility still exists that latent human infection can occur. Fierer et al. [1973] describe the identification and treatment of herpes B virus disease in a virologist whose last direct contact with monkeys had occurred ten years previously. Scinicariello et al. [1993a] identified herpes B virus in a neurobiologist with no recollection of recent exposure to contaminated macaque tissue. The ideal vaccine should not only prevent disease but should also prevent the establishment of latent infections to preclude transmission during a recurrent episode. The candidate vaccine described here is the first step towards achieving this objective.

There is scope to use vBVgD as a veterinary vaccine to prevent infection from imported animals. Herpes B virus free colonies have been established by screening and exclusion of infected macaques, although the majority of non-human primates used for biomedical research are still imported and many are still wild caught. Herpes B virus infection is generally acquired by macaques after puberty [Zwartouw & Boulter, 1984; Weigler et al., 1995] and thus there is the opportunity to protect young macaques from infection with an effective vaccine. Vaccination could play an important role in the long-term goal of developing herpes B virus free colonies.

The question of whether a vaccinia-based vaccine would be licensed for human immunisation in the modern era still remains to be answered. Herpes B virus remains a hazard for monkey handlers because, despite the development of herpes B virus free monkey colonies, significant numbers of herpes B virus seropositive animals are still being used for biomedical research. vBVgD was constructed using the WR strain of vaccinia. This strain is used for research purposes only and would never be licensed for human use because of neurotropic characteristics. Before further animal studies are conducted, a similar recombinant virus should be made using a highly attenuated strain of vaccinia which would be more acceptable for use in humans. Such strains include Lister, which was used for wide scale vaccination against smallpox, NYVAC [Tartaglia et al., 1992] which contains specific genome deletions, or MVA [Mayr et al., 1975] which is also highly attenuated.

As herpes B virus does not occur naturally in humans, and the number of monkey handlers likely to be exposed to the disease is minimal, a phase II vaccine trial is likely to be prolonged and a phase III trial would probably never take place. It is unlikely that a candidate herpes B virus vaccine would ever be licensed for human use, but would remain an experimental vaccine for use by monkey handlers. However, if protective antigens from herpes B virus were incorporated into a multivalent vaccine, the benefit-to-risk ratio of vaccination would increase and the demand for such a vaccine would be greater. For example, a vaccine for simultaneous immunisation against the filoviruses and herpes B virus could be of use for monkey handlers and such a vaccine may also have more widespread application among groups such as research workers and travellers.

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REFERENCES

Advisory Committee on Dangerous Pathogens. 1995. Categorisation of biological agents according to hazard and categories of containment. 4th Edition. London: HMSO.

Bennett AM, Harrington L, Kelly DC. 1992. Nucleotide sequence

analysis of genes encoding glycoproteins D and J in simian herpes B virus. J Gen Virol 73:2963–2967.

Blacklaws BA, Krishna S, Minson AC, Nash AA. 1990. Immunogenicity of herpes simplex virus type 1 glycoproteins expressed in vaccinia virus recombinants. Virology 177:727–736.

Blacklaws BA, Nash AA. 1990. Immunological memory to herpes simplex virus type 1 glycoproteins B and D in mice. J Gen Virol 71: 863–871.

Boulter EA, Kalter SS, Heberling RL, Guajardo JE, Lester TL. 1982. A comparison of neutralisation tests for the detection of antibodies to Herpesvirus simiae (Monkey B virus). Lab Anim Scie 32:150–152.

Boulter EA, Zwartouw HT, Thornton B. 1981. Postexposure immunoprophylaxis against B virus (Herpesvirus simiae) infection. Br Med J 283:1495–1497.

Bourne N, Milligan GN, Schleiss MR, Bernstein DI, Stanberry LR. 1996. DNA immunization confers protective immunity on mice challenged intravaginally with herpes simplex virus type 2. Vaccine 14:1230–1234.

Bourne N, Stanberry LR, Bernstein DI, Lew D. 1996a. DNA immunization against experimental genital herpes simplex virus infection. J Infect Dis 173:800–807.

B Virus Working Group. 1988. Guidelines for prevention of Herpesvirus simiae (B virus) infection in monkey handlers. J Med Primatol 17:77–83.

Cantin EM, Eberle R, Baldick JL, Moss B, Willey DE, Notkins AL, Openshaw H. 1987. Expression of herpes simplex virus (HSV-1) glycoprotein B by a recombinant vaccinia virus and protection of mice against lethal HSV-1 infection. Proc Natl Acad Sci USA 84: 5908–5912.

Carroll M. 1993. Expression analysis and immunogenicity of human immunodeficiency virus type 1 envelope glycoprotein in vaccinia virus. Ph.D. thesis, Faculty of Medicine, University of Manchester.

Chabalgoity JA, Khan CMA, Nash AA, Hormaeche CE. 1996. A Salmonella typhimurium htrA live vaccine expressing multiple copies of a peptide comprising amino acids 8–23 of herpes simplex virus glycoprotein D as a genetic fusion to tetanus toxin fragment C protects mice from herpes simplex virus infection. Mol Microbiol 19:791–801.

Chou Q, Russell M, Birch DE, Raymond J, Bloch W. 1992. Prevention of PCR mis-priming and primer dimerization improves low copy number amplifications. Nucleic Acids Res 20:1717–1723.

Corey L. 1997. Developing a genital herpes simplex virus vaccine. In: Abstracts of the 37th Interscience Conference on Antimicrobial Agents and Chemotherapy, Toronto, Canada. Washington: American Society for Microbiology.

Cremer KJ, Mackett M, Wohelenberg C, Notkins AL, Moss B. 1985. Vaccinia virus recombinant expressing herpes simplex virus type 1 glycoprotein D prevents latent herpes in mice. Science 228:737–740.

Cropper LM, Lees DN, Patt R, Sharp IR, Brown D. 1992. Monoclonal antibodies for the identification of herpesvirus simiae (B virus). Arch Virol 123:267–277.

DiGiacomo RF. 1988. Guidelines for prevention of Herpesvirus simiae (B virus) infection in monkey handlers (letter). Lab Anim Scie 38:558.

Eberle R, Black D, Hilliard JK. 1989. Relatedness of glycoproteins expressed on the surface of simian herpesvirus virions and infected cells to specific HSV glycoproteins. Arch Virol 109:233–252.

Eberle R, Black D, Lipper S, Hilliard JK. 1995. Herpesvirus papio 2, an SA8-like α-herpesvirus of baboons. Arch Virol 140:529–545.

Eberle R, Hilliard J. 1995. The simian herpesviruses. Infect Agents Dis 4:55–70.

Eberle R, Mou SW. 1983. Relative titers of antibodies to individual polypeptide antigens of herpes simplex virus type 1 in human sera. J Infect Dis 148:436–444.

Fierer J, Bazeley P, Brand AI. 1973. Herpes encephalomyelitis presenting as ophthalmic Zoster. Ann Intern Med 79:225–228.

Freifield AG, Hilliard J, Southers J, Murray M, Savarese B, Schmitt JM, Straus SE. 1995. A controlled seroprevalence survey of primate handlers for evidence of asymptomatic Herpes B virus infection. J Infect Dis 171:1031–1034.

Harlow E, Lane D. 1988. Antibodies—A laboratory manual. New York: Cold Spring Harbor Laboratory Press.

Harrington L, Wall LVM, Kelly DC. 1992. Molecular cloning and physical mapping of the genome of simian herpes B virus and comparison of genome organization with that of herpes simplex virus type 1. J Gen Virol 73:1217–1226.

Holmes GP, Chapman LE, Stewart JA, Straus SE, Hilliard JK, Davenport DS and the B Virus Working Group. 1995. Guidelines for the prevention and treatment of B-virus infections in exposed persons. Clin Infect Dis 20:421–439.

Holmes GP, Hilliard JK, Klontz KC, Rupert AH, Schindler CM, Parrish E, Griffin DG, Ward GS, Bernstein ND, Bean TW, Ball MR, Brady JA, Wilder MH, Kaplan JE. 1990. B virus (Herpesvirus simiae) infection in humans: Epidemiological investigation of a cluster. Ann Intern Med 112:833–839.

Hull RN, Nash JC. 1960. Immunisation against B virus infection, I. Preparation of an experimental vaccine. Am J Trop Med Hyg 71: 15–18.

Hull RN. 1971. B virus vaccine. Lab Anim Scie 21:1068–1071.

Hull RN, Peck FBJr, Ward TG, Nash JC. 1962. Immunisation against B virus infection, II. Further laboratory and clinical studies with an experimental vaccine. Am J Trop Med Hyg 76:239–251.

Isaacs SN, Kotwal GJ, Moss B. 1990. Reverse guanine phosphoribosyltransferase selection of recombinant vaccinia viruses. Virology 178:626–630.

Lees DN, Baskerville A, Cropper LM, Brown DWG. 1991. Herpesvirus simiae (B virus) antibody response and virus shedding in experimental primary infection of cynomolgus monkeys. Lab Anim Scie 41:360–364.

Mackett M, Smith GL, Moss B. 1984. General method for production and selection of infectious vaccinia virus recombinants expressing foreign genes. J Virol 49:857–864.

Mayr A, Hochstein-Mintzel V, Stickl H. 1975. Abstammung, Eigenschaften und verwendung des attenuierten vaccinia-stammes MVA. Infection 3:6–14.

McCarthy K, Tosolini FA. 1975. A review of primate herpes viruses. Proc R Soc Med 68:145–150.

McClements WL, Armstrong ME, Keys RD, Liu MA. 1996. Immunization with DNA vaccines encoding glycoprotein D or glycoprotein B, alone or in combination, induces protective immunity in animal models of herpes simplex virus-2 disease. Proc Natl Acad Sci USA 93:11414–11420.

McKenzie R, Straus SE. 1996. Vaccine therapy for herpes simplex virus infections: An historical perspective. Rev Med Virol 6:85–96.

Morgan AJ, Mackett M, Finerty S, Arrand JR, Scullion FT and Epstein MA. 1988. Recombinant vaccinia virus expressing Epstein-Barr virus glycoprotein gp340 protects cotton top tamarins against EB virus-induced malignant lymphomas. J Med Virol 25:185–195.

Petrovskis EA, Timmins JG, Armentrout MA, Marchioli CC, Yancey RJJr, Post LE. 1986. DNA sequence of the gene for pseudorabies virus gp50, a glycoprotein without N-linked glycosylation. J Virol 59:216–223.

Roizman B, Desrosiers RC, Fleckenstein B, Lopez C, Minson AC, Studdert MJ. 1992. The family Herpesviridae: An update. Arch Virol 123:425–559.

Scinicariello F, Eberle R, Hilliard JK. 1993. Rapid detection of B virus (Herpesvirus simiae) DNA by polymerase chain reaction. J Infect Dis 168:747–750.

Scinicariello F, English WJ, Hilliard J. 1993a. Identification by PCR of meningitis caused by herpes B virus. Lancet 341:1660–1661.

Simian Virus Committee. 1985. The management of simians in relation to infectious hazards to staff. Medical Research Council, London.

Slomka MJ, Brown DWG, Clewley JP, Bennett AM, Harrington L, Kelly DC. 1993. Polymerase chain reaction for detection of Herpesvirus simiae (B virus) in clinical specimens. Arch Virol 131:89–99.

Slomka MJ, Brown DWG. 1995. PCR for the detection of herpesvirus simiae (B virus) in clinical specimens. In: Becker Y, Darai G, editors. PCR: Protocols for diagnosis of human and animal virus diseases. Berlin: Springer Verlag. p 253–264.

Slomka MJ, Ashley RL, Cowan FM, Cross A, Brown DWG. 1995a. Monoclonal antibody blocking tests for the detection of HSV-1- and HSV-2-specific humoral responses: Comparison with Western blot assay. J Virol Methods 55:27–35.

Slomka MJ, Harrington L, Arnold C, Norcott JPN, Brown DWG. 1995b. Complete nucleotide sequence of the herpesvirus simiae glycoprotein G gene and its expression as an immunogenic fusion protein in bacteria. J Gen Virol 76:2161–2168.

Tartaglia J, Cox WI, Taylor J, Perkus M, Riviere M, Meignier B,

Paoletti E. 1992. Live vectors as vaccines: Highly Attenuated Poxvirus Vectors. AIDS Res Hum Retroviruses 8:1445–1447.

Tikoo SK, Fitzpatrick DR, Babiuk LA, Zamb TJ. 1990. Molecular cloning, sequencing, and expression of functional bovine herpesvirus 1 glycoprotein gIV in transfected bovine cells. J Virol 64:5132–5142.

Van Drunen Littel-Van Den Hurk S, Babiuk LA. 1986. Synthesis and processing of bovine herpesvirus 1 glycoproteins. J Virol 59:401–410.

Vizozo AD. 1975. Latency of herpes simiae (B virus) in rabbits. British J Exp Pathol 56:489–494.

Wall LVM, Zwartouw HT, Kelly DC. 1989. Discrimination between twenty isolates of *Herpesvirus simiae* (B virus) by restriction enzyme analysis of the viral genome. Virus Res 12:283–296.

Wachsman M, Aurelian L, Smith CC, Lipinskas BR, Perkus ME, Paoletti E. 1987. Protection of guinea pigs from primary and recurrent herpes simplex virus (HSV) type 2 cutaneous disease with vaccinia virus recombinants expressing HSV glycoprotein D. J Infect Dis 155:1188–1197.

Ward JA, Hilliard JK. 1994. B virus specific pathogen-free (SPF) breeding colonies of macaques: Issues, surveillance, and results in 1992. Lab Anim Scie 44:222–228.

Weigler BJ. 1992. Biology of B virus in macaque and human hosts: A review. Clin Infect Dis 14:555–567.

Weigler BJ, Scinicariello F, Hilliard JK. 1995. Risk of venereal B virus (Cercopithecine herpesvirus 1) transmission in rhesus monkeys using molecular epidemiology. J Infect Dis 171:1139–1143.

Zwartouw HT, Boulter EA. 1984. Excretion of B virus in monkeys and evidence of genital infection. Lab Anim 18:65–67.